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ABSTRACT

The release of trophic substances at the neuromuscular junction was studied in nerve-muscle preparations stimulated under a variety of conditions. Experimental conditions, in which synaptic activity was maximized, included electrical stimulation of the nerve in curarized and non-curarized nerve-muscle preparations and treatment with super-physiological concentrations of potassium ion. Control preparations, in which synaptic activity was minimized, included unstimulated nerve-muscle preparations and preparations stimulated while treated with super-physiological concentrations of magnesium ion. The efflux collected in the synaptically active preparations was effective in maintaining acetylcholinesterase levels of denervated muscle in organ culture, and electrophoretic profiles of the synaptically active effluxes showed intensification of several bands, most prominently in the molecular weight range between 30,000 and 40,000 Daltons.

INTRODUCTION

It has been well established that the structure and function of muscle is profoundly dependent on its nerve supply (24). The effects of denervation have been recognized for many years. Neurosurgeons, for example, described in detail muscle atrophy following nerve injuries sustained during the American Civil War (49). More recently, the morphological, biochemical, and electrophysiological changes observed in the denervated muscle have been extensively catalogued (24).

One of the early theories of denervation atrophy maintained that inactivity of the denervated muscle was the primary factor in its degeneration. Electrical stimulation of the denervated muscle has traditionally been used clinically to slow the rate of denervation atrophy (60), and electrotherapy remains the most effective method of influencing the rate of these degenerative changes (24). At best, however, electrotherapy appears to merely retard the onset of denervation atrophy (24). The fact that an intact blood supply and muscle activity alone are not sufficient for the maintenance of normal muscle suggests that the nerve itself has properties which are necessary for muscle integrity. Such properties have been called "trophic" functions. Guth (1968) defines neuromuscular trophic influences as functions of the nerve that regulate the metabolism of the muscle cell.

The concept of the nervous system as a trophic organ is not new. The physiologist Prochaska asked in 1784 "Are nerves necessary for nutrition?", noting that:

> As is known numerous physiologists, old and new, have maintained that nutrition is mediated by nerves. Fr. Sylvius, Willis, Glisson and others have assumed two kinds of nerve fluids: a dense, protein fluid which serves nutritional processes. The other vaporizing,

> more liquid, diffused in the former and destined for eliciting motion and sensation.

Modern neuroscience has provided a more developed explanation for the transmission of nerve impulses. Concepts such as electrochemical gradients, action potentials, the synapse, neurotransmitters, and receptors have replaced Prochaska's poetic concept of a "vaporizing" liquid in explaining the transmission of sensory and motor information in the nervous system. Prochaska's description of "a dense protein fluid which serves nutritional processes" is, however, surprisingly current when considering modern evidence for trophic functions of the nervous system.

Modern experimental evidence for the existence of neurotrophic functions on muscle perhaps began in 1937 when Tower isolated motor neurons and their target muscle by dorsal rhizotomy and spinal cord transection rostral and caudal to a section of cord (67). She noted that certain denervation changes such as early proliferation of nuclei and degeneration of endplate nuclei were more pronounced in the denervated than in the inactivated muscle. Tower concluded that muscle atrophy may result from disuse as well as the cessation of trophic effects of nerve on muscle.

During the 1930's it was also discovered by Torrey that tastebuds degenerated more quickly when the gustatory nerve was transected nearer the buds than when the nerve was transected further away. A similar phenomenon was observed in several subsequent studies which demonstrated that the onset of degenerative changes in muscle was dependent on the length of the peripheral nerve stump after transection (8, 16, 17, 25, 68). Specific dystrophic changes found to be dependent on nerve stump length included: end plate degeneration (25, 68), proteolytic activity (26), glycogen loss (25), fall in acetylcholinesterase activity (17),

acetylcholine hypersensitivity (16, 41), and failure of neuromuscular transmission (41).

Another experimental approach has been to interrupt the peripheral nerve impulse while preserving the anatomical continuity of the axon. Denny-Brown and Brenner (1944) produced motor paralysis by pressure conduction block and found that atrophy of the paralyzed muscle did not take place. These findings, which have been confirmed by others (24, 65), suggest that axoplasmic continuity is sufficient to prevent atrophy in inactive muscle. Studies exploiting the effects of vinblastine and colchicine on microtubules provide evidence that axoplasmic flow transports trophic substances (29, 33, 69).

Perhaps the strongest evidence for the existence of trophic factors comes from experiments in which trophic substances have been extracted from a variety of neural tissues including brain, spinal cord, and peripheral nerve. The trophic actions of these factors have included regulation of AChE (36-39, 53, 55, 59), ACh receptor induction and aggregation (2, 5, 6, 30, 56), and stimulation of morphologic development in muscle cell cultures (46, 47, 52-54, 57). If such substances are synthesized in the nerve cell body, they could theoretically by transported by axoplasmic flow to the synaptic region, where they could be released at the neuromuscular junction and taken up by target muscle cells. The possibility that substances may follow this pathway has been verified by Korr and co-workers who applied substances labelled with P^{32} inorganic phosphate and C^{14} amino acids to the hypoglossal nucleus in the rabbit. They found that the labelled material was transported along the length of the nerve and then incorporated into muscle cells. Also, Younkin and Younkin (1978) reported that myotrophic substances moved by axonal transport were released during nerve stimulation.

The question addressed by the experiments presented here is: If neurotrophic substances are released at the neuromuscular junction during synaptic activity, can such substance be isolated and identified in vitro from nerve-muscle preparations? In this study, nerve-muscle preparations from frogs were stimulated in an amphibian Ringer's solution composed of electrolytes and glucose in physiological concentrations. The bathing solution (efflux) from a number of preparations was pooled, concentrated, and protein content determined. Efflux was also obtained from nerve-muscle preparations after paralysis with curare (to control for effects of muscle contraction), and from preparations treated with 115 mM K^+ to induce synaptic vesicle release (21). Control efflux was obtained from unstimulated nerve-muscle preparations and from preparations which were stimulated while incubated in 20 mM Mg^{++}, which inhibits synaptic vesicle release (10).

Experimental and control effluxes were assayed for trophic activity with respect to maintenance of acetylcholinesterase levels of denervated newt muscle after one week in organ culture, and protein content was characterized by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. Efflux from stimulated and K^+ treated preparations was found to have trophic activity when compared to unstimulated effluxes, and several protein bands in the molecular weight range 30,000 to 40,000 D. were found on gel electrophoresis to be intensified in stimulated effluxes.

MATERIALS AND METHODS

-Collection of stimulated and unstimulated nerve-muscle efflux.

The sartorius muscle and its intact nerve supply were carefully dissected under microscopic visualization from adult medium-sized Rana pipiens of either sex. The nerve and muscle from both legs of each frog

were placed in separate dishes containing amphibian Ringer's solution (118 mM Na^+, 120.6 mM Cl^-, 2 mM K^+, 1.8 mM mM Ca^{++}, 3 mM $H_2PO_4^-$, 3 mM glucose) at pH of 7.4. The bathing solution of both muscles was changed several times to wash off superficial protein before collection of efflux. The proximal end of the experimental preparation was draped across a pair of microelectrodes. The nerve was stimulated at a frequency of 6 sec^{-1} and minimal voltage necessary to produce muscular contraction. When failure occurred, the electrodes were advanced distally along the nerve until contractions could be produced again. This process was continued until the electrodes had been advanced to within several mm of the muscle, or until failure of the muscle occurred. Each experimental preparation could be stimulated in this fashion for 30 minutes or longer, with some preparations lasting up to 60 minutes. Control preparations were unstimulated and left in bathing solution for the same period as stimulated preparations.

-Collection of curarized, stimulated nerve-muscle efflux.

The above protocol was used, with the exception that prior to repetitive stimulation, the muscle was curarized using a .1% d-tubocurarine solution in amphibian Ringer's. The curare dose was titrated under microscopic visualization using periodic test stimuli until minimal contraction of individual muscle fibers could be seen. The muscle was then stimulated repetitively as described above.

-Collections of high K^+ nerve-muscle efflux.

Nerve-muscle preparations were treated with a solution containing 116 mM K^+ (115 mM K proprionate, 1.8 mM Ca proprionate, 1 mM KOH, and 3 mM HEPES buffer at pH 7.4). Muscles were incubated for 30 minutes and efflux was collected. In these preparations, the nerve was not stimulated.

-Collection of high Mg^{++} nerve-muscle efflux.

Nerve-muscle preparations were placed in a Ringer's solution containing 20 mM Mg^{++}, and the nerve was stimulated as described above.

-Storage of samples.

Efflux from each muscle was added to a frozen pool at the completion of each preparation. This pool was stored at -20°C.

-Concentration of Efflux.

Bathing solutions from 10-12 frogs for each experiment was thawed and concentrated by Savant Speed Vac concentrator (model SVC-100H) followed by Amicon Micro-ultrafiltration system (model 8MC) using the UM 10 filter (which retains species of molecular weight 10,000). Samples were concentrated approximately 10X.

-Determination of protein content.

Small samples (20-40 ul) of each concentrated sample were assayed for total protein content according to the photometric method of Lowry (40).

-Bioassay for trophic activity.

The ability of samples to sustain acetylcholinesterase levels in denervated muscle was assayed according to the method of Lentz (1971). Adult newts (Triturus viridescens) of medium size and either sex (Lee's Newt Farm, Oak Ridge, Tennessee) were used. By sterile technique, triceps muscle from each arm was dissected and placed in organ culture dishes. Control medium for one muscle of each pair consisted of stock amphibian culture medium of Wolf and Quimby (72), obtained from Grand Island Biological Co. (Grand Island, N.Y.). The opposite muscle was incubated with Wolf and Quimby media to which samples of concentrated efflux was added. Penicillin (50 u/ml) and streptomycin (50 ug/ml) were added to all stock media. Both control and experimental culture media

was passed through Millipore filters (.22 um pore size) before addition to culture dishes. The culture media was changed after 3-4 days. At the end of one week, muscle acetylcholinesterase levels were assayed according to the photometric method of Ellman (15). Each muscle was weighed and homogenized in phosphate buffer, and a small volume of homogenate was then added to a cuvette containing phosphate buffer and dithiobisnitrobenzoic acid. The substrate, acetylthiocholine iodide, was then added. Activities of acetylcholinesterase in the reaction mixtures were then determined by reading changes in absorbance at 412 nm over a period of 10 minutes.

-Polyacrylamide gel electrophoresis.

Protein composition of concentrated effluxes were analyzed by SDS polyacrylamide gel electrophoresis according to the method of Maizel (44). A gel slab was prepared using 5% acrylamide stacking gel and 12% acrylamide resolving gel. Samples containing equal amounts of protein (80 ug) in volumes up to 100 ul in each lane were applied to the gels. A sample of proteins of known molecular weights served as standards. After electrophoresis, gels were stained with Coomasie blue 250-R, destained with acetic acid, and then photographed. The molecular weights of individual bands were determined by comparison with the mobility of standards as described by Weber and Osborn (70).

RESULTS

The average protein released by each nerve-muscle preparation as determined by photometric assay after concentration is reported in Table 1. The experimental preparations, in which synaptic activity was maximized by either electrical stimulation of the nerve or by treatment with high K^+, released two to three times more protein than did control

Table 1. Protein Release From Nerve-Muscle Preparations

Preparation	N (no. of muscles)	Average total protein/preparation
Control	12	12 ug
Mg^{++}	10	10 ug
Stimulated	14	27 ug
K^{+}	12	30 ug
Curare	10	25 ug

Table 2a. Acetylcholinesterase Activity in Untreated Muscles

Sample	N (pairs)	L*	R*	Δ
Uncultured muscles	6	4.23	4.26	+1%
Cultured muscles, untreated	8	4.33	4.29	-1%

Table 2b. Acetylcholinesterase Activity in Treated Muscles**

Sample	N (pairs)	Experimental*	Control*	Δ	$\Delta \pm$ s.e.m.	P (t test)
Control efflux	6	4.07	4.05	+1%	$+.02 \pm .21$	$>.05$
Mg^{++} efflux	6	4.05	4.08	-1%	$-.03 \pm .37$	$>.05$
K^{+} efflux	12	4.33	4.02	+7%	$+.31 \pm .14$	$<.05$
Stim. efflux	14	4.12	3.75	+9%	$+.34 \pm .15$	$<.05$
Curarized efflux	10	3.95	3.59	+10%	$+.32 \pm .14$	$<.05$

*Mean values for AChE rates (umol of substrate hydrolyzed per min. per gm. muscle).

**One muscle of each newt arm pair was treated with efflux of concentration 8-12 ug protein per ml medium. The other muscle was untreated and served as a control. Medium was changed after 3-4 days, and after one week in culture, AChE of each muscle was assayed. The standard error of the mean (s.e.m.) was calculated for the differences between experimental and control groups ( ) and tested at a .05 significance level by the t-test.

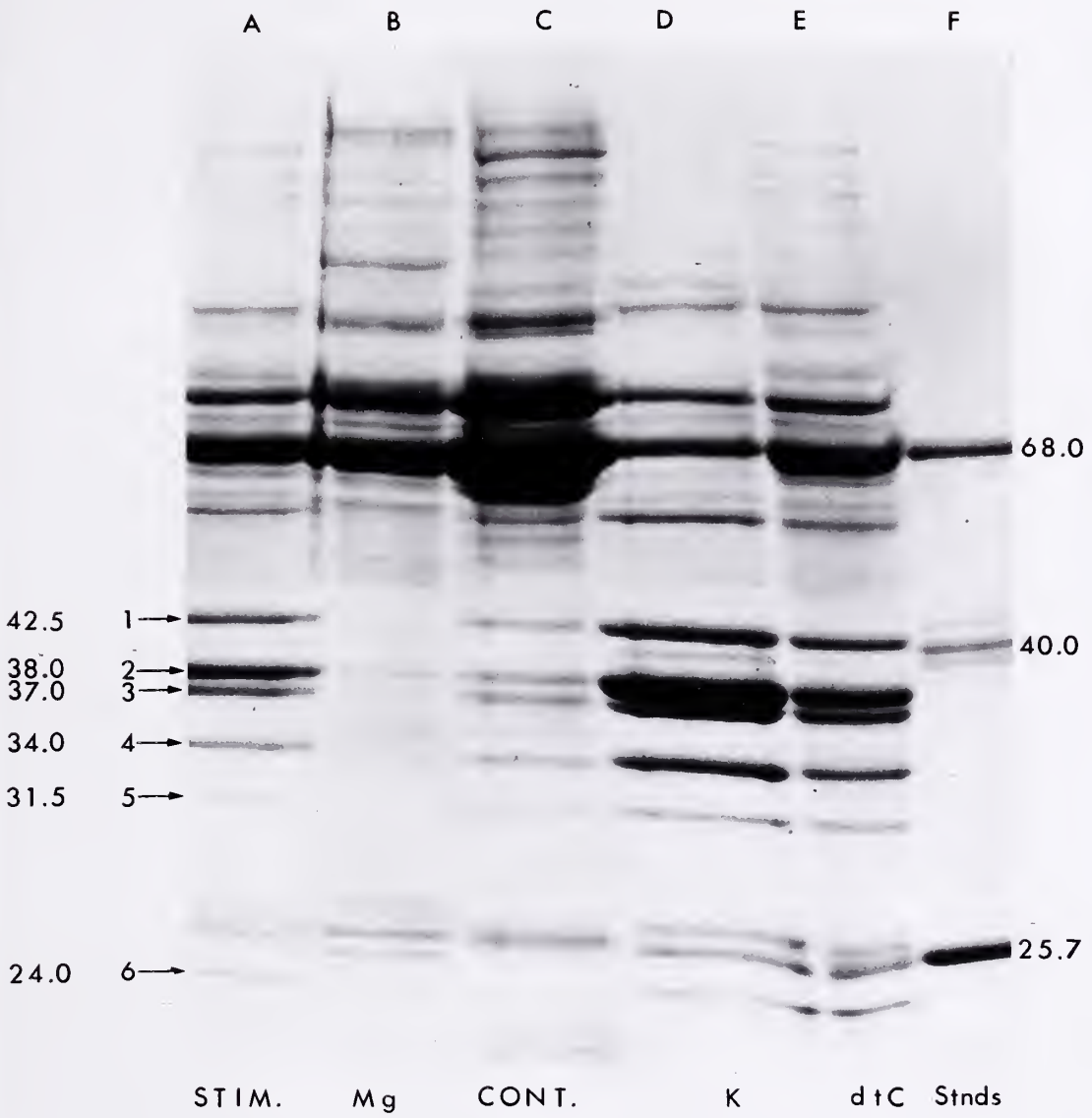
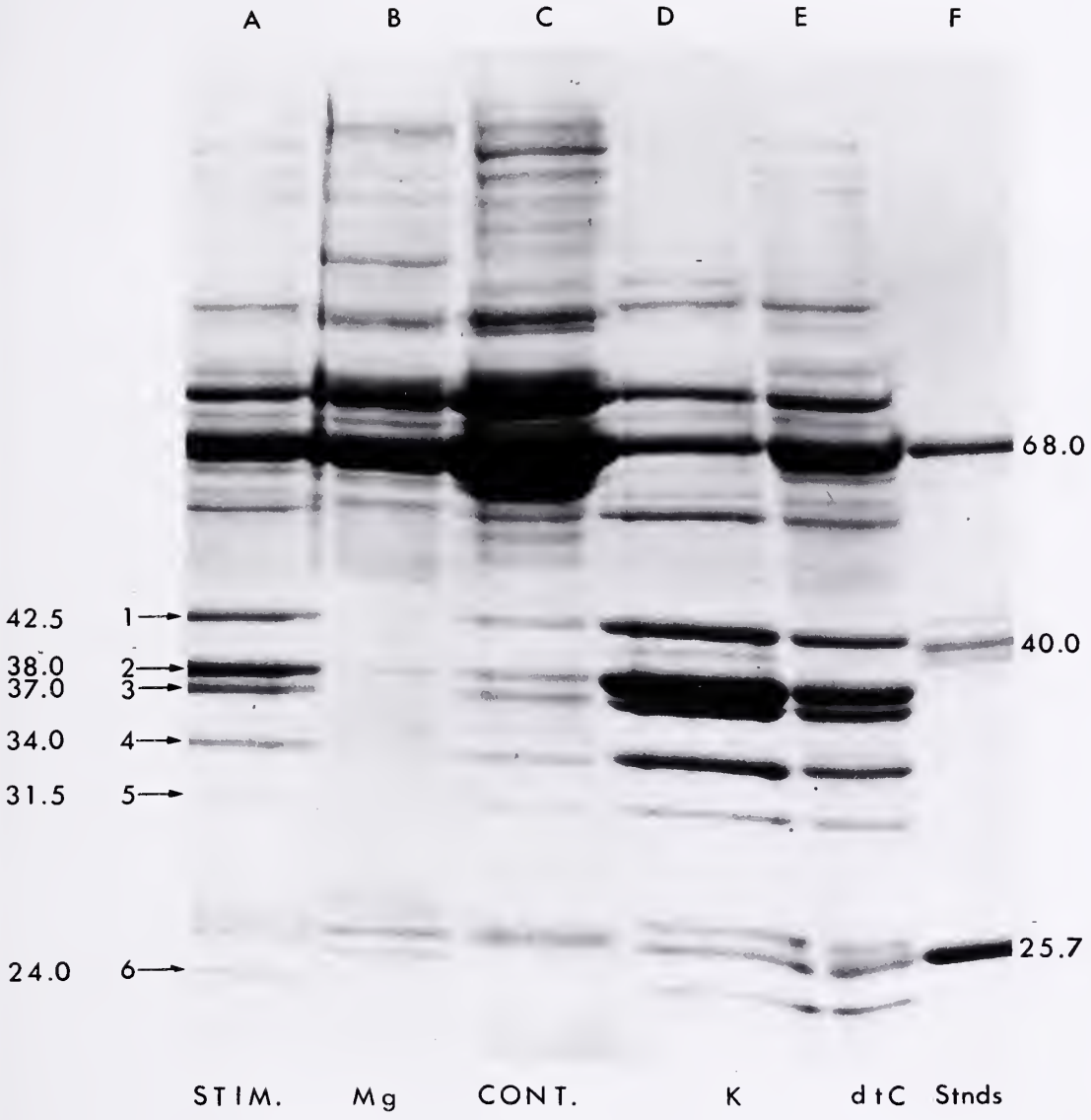


Fig. 1. SDS-polyacrylamide gel profiles of nerve-muscle effluxes.

Equal amounts (80 ug) of total protein were loaded in each lane. Standards (lane F) used were bovine serum albumin (M.W.=68,000), horseradish peroxidase (M.W.=40,000), and a-chymotrypsinogen (M.W.= 25,700). Arrows numbered 1-6 indicate bands which were intensified in synaptically active preparations, and their respective molecular weights are listed to the left.



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preparations of either unstimulated or Mg^{++} treated preparations. Paralysis of the muscle with d-tubocurarine did not significantly reduce protein release from stimulated nerve-muscle preparations.

-Bioassay for trophic activity.

The results of acetylcholinesterase (AChE) assays on untreated muscle (both cultured and uncultured) are shown in Table 2a. AChE activities were the same for untreated right and left arm muscles both before and after culture for one week.

Table 2b summarizes the results of AChE assays on muscle pairs after one week of culture, with one muscle of each pair treated with one of the five groups of efflux, while the other muscle remained untreated. No significant differences in AChE activity were found between muscles treated with efflux from unstimulated or Mg^{++} treated nerve-muscle preparations and untreated muscles. AChE activities in muscles treated with efflux from the synaptically active preparations, however, were higher than in untreated muscles. Treatment with the K^{+} efflux resulted in AChE levels which were 7% higher than controls after one week in culture, and treatment with efflux from the curarized and non-curarized, stimulated preparations resulted in +10% and +9% differences in AChE, respectively. These differences are comparable to the results of others using this assay (39, 59). The relatively small changes are due to the fact that a considerable portion of the muscle AChE is unaffected by reinnervation (39). When the molecular forms of AChE are examined, it is found that the greatest changes in response to trophic influences occur in the 16S end plate specific form of the enzyme (Lentz et al, '81).

-Polyacrylamide gel electrophoresis.

Gel electrophoresis of equal amounts of total protein from concentrated effluxes resulted in the profiles shown in Figure 1 (p. 9). The

synaptically active effluxes (lanes A, D, and E) contain several bands (labelled 1-6) which are intensified in comparison to the control efflux (lane C). The most prominent differences appear in the bands of apparent molecular weights 38,000 and 34,000 (bands 2 and 3). Bands 1-6 are less intense in the Mg efflux (land B). Bands 1, 3, and especially band 2 are most prominent in the K efflux.

DISCUSSION

In this study substances obtained from stimulated nerve-muscle preparations maintained AChE levels in denervated muscle organ cultures, and electrophoretic profiles of these effluxes showed several bands which were more intense than in profiles of biologically inactive effluxes obtained from unstimulated preparations. Treatment of nerve-muscle preparations with K^{+} yielded effluxes with bioactivity and electrophoretic profiles demonstrating intensification of the same bands. Treatment with Mg^{++} resulted in a reduction of intensity of these bands and elimination of bioactivity. These results demonstrate that electrophoretic profiles of myotrophic effluxes from synaptically active nerve-muscle preparations may differ from biologically inactive preparations in which synaptic activity is suppressed.

There may be several factors contributing to the electrophoretic profiles of each of the effluxes. All preparations contained substances with migration patterns characteristic of albumin, pre-albumin, and a spectrum of immunoglobulins. This pattern, seen in the superior third of the gel, indicates that leakage of serum proteins into the efflux was one source of protein. This seems quite likely, since even with careful dissection, some microvascular trauma is unavoidable. Trauma to muscle tissue during dissection may also result in the release of intracellular

proteins, such as the glycolytic enzymes (9). Another possibility is that anoxia due to hypoperfusion may alter muscle membrane permeability and therefore result in protein release (75). The nerve may have also been a source of protein in these effluxes, either by passive diffusion of substances out of the nerve and/or by selective release of substances at the neuromuscular junction. Therefore, the profile of the control efflux represents a composite of proteins from serum, muscle, and nerve.

Curarization of the muscle prior to nerve stimulation acted as a control for the effects of post-synaptic depolarization. These would include mechanical and metabolic effects of muscle contraction on efflux composition. The intensities of bands 1-6 in the profile of the curare efflux are comparable to the stimulated efflux profile, which suggests that the release of the protein in these bands was caused by nerve stimulation and not by muscle activity.

The increased intensities of bands 1-6 in the profile of the K^+ efflux could be the result of altered muscle membrane permeability, thereby allowing increased diffusion of proteins into the efflux (75). Alternatively, protein release from nerve may have been stimulated by K^+, which has been shown to cause depletion of synaptic vesicles and subsequent extinction of MEPPs after application to the neuromuscular junction (21). The fact that the bands which are intensified in the K^+ efflux profile are reduced in intensity in the Mg^{++} efflux profile suggests that these ions had antagonistic effects on protein release. Mg^{++} is known to suppress synaptic vesicle release at the neuromuscular junction (10), therefore the proteins in bands 1-6 may have been released from synaptic vesicles.

Results of the AChE assays indicate that trophic substance concentrations were greater in effluxes from stimulated preparations.

Furthermore, the antagonistic effects of Mg^{++} and K^{+} suggest synaptic vesicles may be involved in release of trophic substance. The greatest changes in intensity occurred in the bands corresponding to proteins of molecular weight 30,000 to 40,000. It is likely that some of the bands showing differences in stimulated preparations may represent proteins which have functions unrelated to myotrophic regulation. However, AChE-regulating proteins of similar molecular weights have been isolated from nerve homogenates (39, 46). In particular, a factor regulating ChE activity and isolated from brain (Lentz et al, '81) corresponds to band 2 in the stimulated effluxes. On the other hand, some myotrophic proteins with considerably different molecular weights than these have been isolated (34, 47, 52, 56, 57). It is probable that there are several discrete myotrophic proteins, each with its own regulatory mechanism. For example, in addition to factors regulating AChE, other factors may regulate ACh receptor synthesis and distribution (2, 6, 62). Not all of these factors may be released concomitant with synaptic vesicle discharge. Also, several species may be involved in the regulation of each trophic effect, such as certain factors which suppress muscle AChE (36, 59).

As evidence suggesting the existence of neuro-myotrophic substances accumulates, it becomes possible to construct hypotheses regarding the synthesis, transport, and release of such substances. It seems likely that trophic proteins are synthesized in the nerve cell body, since most neuronal protein synthesis has been shown to occur in the perikaryon (14). This would explain the presence of trophic proteins in central nervous system tissue as well as peripheral nerves, since the cell bodies for peripheral nerves lie within the CNS.

The transport of myotrophic substances has been investigated using colchicine treatment of peripheral nerves (1, 4, 28, 33, 69). Colchicine apparently combines with and disrupts the structure of microtubules, which are involved with a variety of intracellular transport mechanisms. Their presence in neurons (as "neurotubules") is believed to be necessary for the fast component of axoplasmic flow (51). Since denervation-like changes have been demonstrated after treatment of peripheral nerve with colchicine, it has been concluded that trophic substances may be transported by fast axoplasmic flow.

Several possibilities could account for the trophic effects of neurons. One theory is that the neurotransmitter ACh itself has trophic properties. One recent study reported that a-bungarotoxin, which binds irreversibly to ACh receptor, produced denervation-like changes after application to muscle in vivo (12). It has been suggested, therefore, that ACh is responsible for trophic effects either directly by specific post-synaptic action or indirectly through the effects of muscle activity (61). In this case, ACh delivery from synaptic vesicles could occur by active nerve transmission, spontaneous quantal release (18), or by continuous ACh leakage (12, 31, 64).

On the other hand, there is evidence that the release of ACh alone does not account for the entire spectrum of neuromyotrophic functions. Application of ACh has been shown to be ineffective in preventing denervation changes (23, 48), and colchicine-induced denervation changes occur even though colchicine does not interrupt ACh-dependent neuromuscular transmission (23). Therefore, another possibility is that neurotrophic substances in addition to ACh are released at the neuromuscular junction. Protein release with neurotransmitter had been demonstrated in sympathetic nerve endings (63), which led Music and Hubbard (50) to

investigate protein release by cholinergic nerve endings. They found that protein release did occur during stimulation of mouse nerve-muscle preparations, and that certain fractions of this protein bound significant amounts of ACh. It was suggested that these proteins and ACh could have been released together.

Protein release could occur through several mechanisms including release from common synaptic vesicles, release from other vesicular structures, or non-quantal release. Isolated synaptic vesicles contain ACh, ATP, Ca^{2+}, and several proteins including actin. Two other synaptic vesicle proteins are thought to be involved with ATP transport and utilization (a probable ATPase), while others remain unidentified (71). The effects of K^{+} and Mg^{++} on trophic substance efflux and electrophoretic profiles suggest that synaptic vesicles may also contain and release myotrophic proteins. An alternative could be release from other synaptic structures such as large dense core vesicles (LDCVs) in response to nerve terminal depolarization. A reduction of these vesicles by stimulation in frog sartorius nerve-muscle preparations has been reported by Lynch (1980), which suggests that LDCVs may release their contents during synaptic activity. Although no specific function of LDCVs has yet been identified, they could conceivably play a role in protein release by a mechanism similar to that exhibited by neurosecretory granules (3, 27, 43, 73). Since the effects of K^{+} and Mg^{++} on LDCV release are unknown, it is difficult to interpret the effects of these ions with respect to this hypothetical mechanism of release. However, the fact that stimulation results in release of LDCVs suggests that this could be a mechanism of trophic substance release.

The relative contributions of ACh and other substances to myotrophic processes have not been established. Seemingly conflicting evidence

exists, as some authors argue that effects of the neurotransmitter ACh alone are sufficient to account for the trophic influence of nerve on muscle (12, 61), while others have isolated a variety of myotrophic proteins. Both theories, however, must involve the release of the respective trophic agent(s) from the nerve, followed by diffusion across the synaptic cleft to act on target cells. A theory of protein release along with ACh from synaptic vesicles could resolve these conflicting views. Another possibility would be release of trophins from LDCVs, although the effects of K^+ and Mg^{++} on LDCV release remain to be demonstrated.

CONCLUSION

Intensification of several bands in electrophoretic profiles of effluxes from stimulated nerve-muscle preparations has been dmonstrated. These effluxes were also shown to have positive effects on AChE levels in denervated muscle in organ culture. Further experiments will be necessary to determine the identities and functions of the individual proteins in the intensified bands. These proteins seem to be released during synaptic activity, and further isolation of these factors may help resolve the question of whether myotrophic proteins are released from the neuromuscular junction.

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